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The invention refers to a new technique for global measurements of subcellular dynamics of gene expression, proteins, metabolites etc, the spatial distribution of at least one chemical substance retained by a biological matter being analyzed.

Cellular reactions against allogenic materials involve the production of signal mediators in connection with contact with different materials and drugs.

15 For example, the blood reaction to foreign materials may engage several major defense systems, e.g. the coagulation cascade, the complement system, fibrinolysis, the kinins, platelet derived growth factors, platelet chemokines, and leukocyte derived factors, like prostaglandins, lipid peroxidation products or ceramides. Attempts to measure blood reactions to materials by choosing one of these factors will always meet with the possibility that other factors may be more important. Methods available today for measuring cell reactions comprise immunocyto-  
20 chemistry and the like, one pre-determined substance at a time being detected.

25 In connection with for example proteomics it is also desirable to be able to apply general global measurements, whereby a large number of components, for example proteins, can be simultaneously detected in one sample only. Global measurements can explain how proteins, nucleic acids, and small molecules interact with each other to form networks or modules that carry out specific functions. Today, such measurements are integrated, i.e. the measurements are performed in liquid media or cell suspensions, large volumes being required with accompanying complicated separation techniques.

Thus there is a strong demand in the rapidly advancing fields of gene expression acquisition technologies, gene expression data analysis, functional analysis of biological control systems, proteomics, modelling and analysis of kinetic networks, metabolomics, signal transduction, morphogenesis, molecular neurobiology, etc, for a method, whereby it is possible to measure several factors simultaneously, rather than by studying the detailed behaviour of single components. Methods for global measurements on individual cells, including subcellular levels, are not available today.

According to the invention, a method is provided for analyzing the spatial distribution of at least one chemical substance retained by a biological matter. The chemical substance should mainly comprise organic material, which for example can comprise a lipid, an amino acid, a peptide, a protein, a carbohydrate, a nucleotide, a transmitter substance, a drug, or a targeting molecule. The biological matter can for example comprise cells, tissue, virus, body liquid, or biological molecules. Thus, the chemical substance retained by the biological matter can be located within or on the same.

In order to determine the spatial distribution of the chemical substance, a targeting molecule could be arranged to bind to or react with specific targeted moieties of known identity of the biological matter and function as a marker for those molecules which are to be identified. For example, when specific proteins is to be analyzed, antibodies or fragments thereof, which are directed towards specific targeted moieties on the same, can be used as targeting molecules. Similarly, when a specific DNA-sequence of a DNA-molecule is to be analyzed, the targeting molecule is a complementary DNA-sequence to the nucleotide of interest. The targeting molecule can also comprise a chemical label, for example an unusual element or an

isotope, in order to improve the detectability in the analytical technique employed, i.e. when larger molecules (e.g. whole proteins) are to be detected. In this connection an unusual element or isotope means an element or an 5 isotope which is not naturally present or present only in low concentrations in the biological matter analyzed.

The first step in the inventive method is to supply a sample of the biological matter as a specimen surface. Such a sample can be supplied as a specimen of a solid or semi-10 solid material. For example, an *in situ* specimen surface can be used directly, when the healing (ingrowth) of a titanium implant with a structured surface is to be studied.

The method according to the invention is general and 15 can be used directly on complicated specimens, such as for example dialysis membranes after use. For example, peritoneal cells can be analyzed in connection with peritoneal dialysis by supplying the membrane used as specimen surface. Thus, the method is especially useful for studying 20 cell preparations of blood cells on biomaterials. By studying material/blood reactions, information regarding the influence of man-made synthetic products on the cells can be detected directly.

The sample of the biological matter can also be 25 supplied as a specimen surface by applying it on a solid surface, the solid surface being provided as a support for the biological matter. In this case the biological matter is in a more liquid state, such as blood and tissue fluid, but can also be a more delicate matter, such as a frozen 30 tissue section.

The solid surface is generally a glass surface, but can be any other suitable solid surface in dependence on the specific application. This is especially relevant when 35 cells are to be analyzed for adhesion, spreading or chemo-tactic movement.

If necessary, the specimen surface is also prepared by subjecting it to lyophilization, freeze-substitution, or air drying.

According to the invention, an imprint of the specimen surface is then produced on a substrate surface, whereby at least one chemical substance is distributed on the same. In order to be compatible with the analytical technique employed in the inventive method, the substrate surface should be a metal surface. Suitable metal substrate surfaces are silver, gold, palladium, platinum, nickel, chromium, and copper.

In order to improve the imprinting effect, the specimen surface should be pretreated immediately before the imprint is produced. One pretreatment comprises the condensation of liquid of a non-polar solvent and/or a polar solvent onto the specimen surface. Preferably, the polar solvent is a water solution.

The pretreatment can be accomplished by first bringing the specimen surface to room temperature or cooling the same to a lower temperature and then condensing the solvent vapor thereon by arranging the specimen above a heated container containing the liquid. The imprint should be produced within 10 s after the pretreatment of the specimen surface.

Likewise, the substrate surface should be polished and/or cleaned immediately before the imprint is produced. Suitable cleaning methods are chemical etching, plasma cleaning, and vaporization deposition. Of course, the cleaning methods can be combined.

Thus, a crucial step in the inventive method is the production of the imprint of the specimen surface on the substrate surface in order to distribute and "immobilize" chemical substance(s) on the same.

The imprint is preferably produced by pressing the specimen surface against the substrate surface. This can be

accomplished by pressing a compressible material against the opposite side of the specimen surface and/or the opposite side of the substrate surface and by applying thereon a force between 0.01 and 10 MPa. The pressing

5 should be performed for up to 100 s.

In this process individual components, such as ions and larger molecules, are transferred to the substrate surface. An imprint of the distribution is obtained, which is dependent on the pretreatment and pressing parameters.

10 The pressing procedure is facilitated by the specimen surface and/or the substrate surface being made of a flexible material.

Likewise, the transfer of chemical substance(s) to the substrate surface is facilitated by the substrate

15 surface being structured. Preferably, the substrate surface is structured with protrusions of 0.01-5 µm in width and/or length.

According to the invention, the imprint is then subjected to imaging mass spectrometry, wherein at least one

20 signal is produced from at least one point of the substrate surface. The magnitude of this signal is dependent on the amount of the chemical substance present on the substrate surface.

The at least one signal is then recorded. Preferably,

25 the signal is recorded from an array of points on the substrate surface.

Multiple sequential imprints can also be produced from the same area of the specimen surface. In this case each of the imprints is produced on a separate substrate

30 surface. Then each imprint on each substrate surface is subjected to imaging mass spectrometry.

A suitable imaging mass spectrometry is a secondary ion mass spectrometry. Secondary ion mass spectrometry (SIMS) is a surface analytical technique that has been

employed for spatially resolved analysis of atoms and molecules at the single cell and subcellular levels.

In this connection silver is the preferred substrate surface, since silver is an almost optimal substrate for 5 the analysis of intact molecular ions because of the ability of silver to cationize large molecules.

Thus, when deposited on a clean silver substrate the chemical substance(s) can be cationised by  $\text{Ag}^+$ , peaks in the spectrum being provided which correspond to the mass of 10 the intact molecule plus the  $\text{Ag}^+$  ion  $(\text{M}+\text{Ag})^+$ . A conclusive identification of the detected molecules is then possible. The identification of unknown compounds is aided by spectral matchings with a library.

For the cationization of the chemical substance by 15 substrate ions to occur in SIMS, the chemical substance to be analyzed must not be present on the substrate surface in too large quantities. Thus, the pressing is performed so that the imprint represents below 5 monolayers, preferably below 2 monolayers, of the chemical substance(s) on the 20 substrate surface.

Preferably, a focused beam of ions should be produced by the primary ion source in the secondary ion mass spectrometry, the ions being  $\text{C}_{60}$ , Ga, In, or Au ions. When gold ions are used, they are clusters of  $n$  ions, in which 25  $n \leq 10$ . The focused beam should have a diameter below 10  $\mu\text{m}$ , preferably below 1  $\mu\text{m}$ .

The imaging mass spectrometry can also be a matrix assisted laser desorption ionisation. In this case a light sensitive matrix is applied onto the substrate surface 30 before and/or after the production of the imprint. The light sensitive matrix can be  $\alpha$ -cyano-4-hydroxycinnamic acid, trans-3-indoleacrylic acid, 3-methoxy-4-hydroxy-cinnamic acid, 2,5-dihydroxybenzoic acid, or 3,4-di-hydroxycinnamic acid. The light source of the matrix

assisted laser desorption ionization should comprise a focused laser beam, preferably an ultraviolet laser beam.

At last the distribution of the at least one chemical substance is determined from at least one image of the 5 imaging mass spectrometry. Mass spectra are obtained with high mass resolution as well as images with high lateral resolution. The resolution is between 100 nm and 1  $\mu$ m.

Each image is in turn produced from the signal, the colour or the brightness in each point of the image being 10 dependent on the magnitude of the signal from the corresponding point on the substrate surface. In this way images of chemical distributions are obtained. The analysis as well as the regeneration of images is accomplished by 15 means of advanced information technology, whereby image processing as well as statistics for handling and processing of the large amounts of data is provided.

Preferably, the secondary ion mass spectrometry is time-of-flight secondary ion mass spectrometry (TOF-SIMS). This is a mass spectrometric method with a high lateral 20 resolution of down to 60 nm combined with the ability to measure secondary ions with masses up to at least 10 000 atomic mass units.

This type of secondary ion mass spectrometry is a relatively new technique for chemical surface analysis and 25 it has several advantages compared to other surface analysis methods. Most significantly, TOF-SIMS is the only method which has the potential for spatially resolved identification and chemical analysis of organic molecules on surfaces in the submicrometer range.

30 A TOF-SIMS spectrum is recorded under high vacuum by scanning the primary ion beam over the area of interest on the substrate surface and acquiring a positive or negative mass spectrum of the ions leaving the surface.

**EXAMPLES**

The invention will now be further described and illustrated by reference to the following examples. It should be noted, however, that these examples should not be construed as limiting the invention in any way.

***Example 1. Ion microscopy.***

Ions or other molecules from dried specimens are transferred to and immobilized on a silver surface by 10 carefully pressing a freshly etched silver foil onto the dried specimen surface.

For analysis of organic compounds according to the invention, the accumulated primary ion dose is kept below the so called static limit, which means that the analysis 15 is completed before the analyzed surface has been significantly damaged by the primary ions. In a TOF-SIMS image, the brightness of each pixel reflects the signal intensity of a selected ion in that pixel. The recorded TOF-SIMS spectra are stored in raw data files which contain 20 complete spatial and spectral information from the data collection, thereby allowing for subsequent extraction of images of arbitrary ions and extraction of mass spectra from restricted areas within the analysis area at any time after data collection. Data are collected at either high 25 mass resolution  $m/\Delta m > 7000$  or lateral resolution ( $<100$  nm).

***Example 2. Whole blood in vitro.***

Venous blood from a volunteer is sampled and placed 30 in drops onto different material surfaces and incubated at 37°C in a humid chamber for varied periods of time. The coagulated blood is then gently washed off and the surfaces are allowed to dry in air. Each preparation is washed with distilled water and dried.

35 The result of this procedure is a surface layer of plasma proteins and blood cells. The blood cells adhere and

are activated differently at different surfaces by detecting the cell expression of integrins and selectins.

More specifically, capillary blood was placed in drops on a clean glass surface and incubated for 30 min at 5 37°C. The clot was rinsed off with Dulbeccos phosphate-buffered saline and the saline was removed from the glass surface-adhering cells by a rinse in 0.15M NH<sub>4</sub>COOH at pH 7.2-7.4. The glasses were then placed on a solid copper block pre-cooled with liquid nitrogen in a vacuum chamber 10 that was evacuated down to 10<sup>-4</sup>-10<sup>-5</sup> bar.

**Example 3. Distribution of cell components.**

A clean silver foil is pressed against a glass surface prepared as above and the imprinted silver foil is 15 subsequently analyzed by means of TOF-SIMS at different mass-to-charge ratios of different ions (m/z) with reference to Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>++</sup>, amino acids, and cholesterol, a resolution of less than 0.5 μm being obtained.

20 1) Distribution of m/z=23 (indicative of Na<sup>+</sup>).

In this case the resulting TOF-SIMS images showed platelets with a low internal concentration of Na<sup>+</sup> and leukocytes (see below) with membrane leakage of Na<sup>+</sup>.

25 2) Distribution of m/z=30 (indicative of CH<sub>4</sub>N<sup>+</sup>).

This signal is common for several different amino acids, their presence being established.

30 3) Distribution of m/z=39 (indicative of K<sup>+</sup>).

Platelets exhibit a high internal concentration of K<sup>+</sup>, indicating an intact membrane, whereas the leukocytes exhibit membrane leakage of K<sup>+</sup>.

35 4) Distribution of m/z=40.1 (indicative of Ca<sup>++</sup>).

All cells exhibit a granular distribution of Ca<sup>++</sup>.

5) Distribution of m/z=493.3 and m/z=495.3 (indicative of cholesterol-<sup>107</sup>Ag<sup>+</sup> and cholesterol-<sup>109</sup>Ag<sup>+</sup>, respectively) and

m/z=879.6 and m/z=881.6 (indicative of cholesterol dimer- $^{107}\text{Ag}^+$  and cholesterol dimer- $^{109}\text{Ag}^+$ , respectively),.

These combined distributions resulted in a very reliable localization of cholesterol in the cells studied.

5 Thus, the imaging of subcellular distribution can be demonstrated at a resolution better than 100nm for signals corresponding to  $\text{Na}^+$ ,  $\text{K}^+$ ,  $\text{Ca}^{++}$ , cholesterol and total protein.

10 **Example 4. Cell preparation.**

Three different cell preparation methods have been used, air drying, freeze substitution, and freeze drying. Air drying was performed in saline followed by rinsing with water or volatile buffers to remove salts. The presence of 15 salt always ruined all possibilities to obtain reproducible data. Freeze substitution was performed in ethanol, acid ethanol, methanol, acid methanol, methanol/water 80/20 in various combinations of buffers and volatile salts. The use of solvents, even dilute methanol, always removed cholesterol from the cytoplasmic membranes.

20 The only preparation method that gave reproducible localisation of membrane lipids was freeze drying in volatile salts. Cholesterol, cholesterol dimer and phosphocholine have been localised. Cholesterol and 25 phosphocoline showed different and apparently complementary localisation in surface-adhering leukocytes.

**Example 5. Platelet adhesion and activation.**

30 The adhesion and spreading of platelets on protein-coated surfaces is studied with respect to receptors involved and membrane expression of integrins and selectin. The spreading of cells is often accompanied by changes in membrane composition e.g. the exposure of phosphatidylserine in the outer leaflet seen during apoptosis. Such an 35 exposure of other membrane lipids, also with a short

halftime due to extracellular breakdown, is studied by means of the method according to the invention.

**Example 6. Chemotaxis.**

5 Chemotaxis, defined as the ability of orientation and directed migration in chemical gradients, is a key response of the immune system and a universal cell biological phenomenon. The regulation of this process is complex and not characterized in detail. The compartmentalization of the  
10 intracellular signalling system in chemotaxis is a key issue in understanding the mechanisms that control cell orientation in chemotactic gradients. The spatial intracellular resolution of the cell components provide data with reference to those mechanisms, especially to time resolu-  
15 tion.

In this connection the inositol lipids (PIP2 and PIP3) are important lipid intracellular messengers under study that are involved in the local control of the actin cytoskeleton and they have distinct functions in the local  
20 and global regulation of pseudopode formation. Other lipid mediators, such as diacyl glycerol, are involved in secretory responses, such as degranulation and superoxide release, are also studied.

Glass surfaces are first coated with different  
25 proteins by means of physical adsorption, a routinely used technique. Freshly isolated cells are then incubated at the protein-coated surfaces. For experiments with chemotaxis, special chambers have been constructed for exposure of the cells with a gradient of a chemoattractant. Cell adhesion,  
30 polarisation and spreading is studied by means of the method according to the invention and compared with fluorescence microscopy.

Accordingly, the inventive method ("ion microscopy") can be used as a tool in cell biology and enables the  
35 analysis and localisation of cell signal mediators, like phospholipids, lipid oxidation products, and ultimately

large molecules like whole proteins. The global molecular distribution of individual components within a cell can be reproduced in order to obtain cell specific information on subcellular dynamics of gene expression and proteins.

5        The method is also applicable to cell surface interactions as well as the influence of different drugs on cell reactions. A comparison can be performed before and after the biological matter has been exposed to different environmental factors. In addition, new materials can be  
10      studied, which are developed for the treatment of wounds, dialysis and implants.

## CLAIMS

1. A method of analyzing the spatial distribution of at least one chemical substance retained by a biological matter, characterized by the steps of
  - 5 (a) supplying a sample of said biological matter as a specimen surface;
  - (b) producing an imprint of said specimen surface on a substrate surface, said at least one chemical substance 10 being distributed on the same;
  - (c) subjecting said imprint to imaging mass spectrometry, at least one signal from at least one point of said substrate surface being produced, the magnitude of said at least one signal being dependent on the amount of said at 15 least one chemical substance present on said substrate surface;
  - (d) recording said at least one signal; and
  - (e) determining said distribution of said at least one chemical substance from at least one image of said imaging 20 mass spectrometry.
2. The method as in claim 1, wherein said at least one chemical substance mainly comprises organic material.
3. The method as in claim 2, wherein said organic material comprises a lipid, an amino acid, a peptide, a 25 protein, a carbohydrate, a nucleotide, a transmitter substance, a drug, or a targeting molecule.
4. The method as in claim 3, wherein said nucleotide is a DNA-molecule.
5. The method as in claim 3 and 4, wherein said targeting molecule is a complementary DNA-sequence.
- 30 6. The method as in claim 3, wherein said targeting molecule is an antibody or a fragment thereof.
7. The method as in any of claims 3-6, wherein said targeting molecule comprises a chemical label.
- 35 8. The method as in claim 7, wherein said chemical label is an unusual element or an isotope.

9. The method as in any of claims 1-8, wherein said biological matter comprises cells, tissue, virus, body liquid, or biological molecules.

10. The method as in any of claims 1-9, wherein said 5 sample of said biological matter is supplied as a specimen surface *in situ*.

11. The method as in any of claims 1-9, wherein said sample of said biological matter is supplied as a specimen surface by applying it on a solid surface.

10 12. The method as in claim 11, wherein said solid surface is a glass surface.

13. The method as in any of claims 1-12, wherein 15 multiple sequential imprints are produced from the same area of said specimen surface, each of said imprints being produced on a separate substrate surface.

14. The method as in any of claims 1-13, wherein said specimen surface is pretreated immediately before said imprint is produced.

15. The method as in claim 14, wherein said specimen 20 surface is pretreated by condensing a liquid of a non-polar solvent and/or a polar solvent onto the same.

16. The method as in claim 15, wherein said polar solvent is a water solution.

17. The method as in claim 15 or 16, wherein said 25 specimen surface is first brought to room temperature or cooled and is then arranged above a heated container containing said liquid.

18. The method as in any of claims 14-17, wherein said 30 imprint is produced within 10 s after said pretreatment of said specimen surface.

19. The method as in any of claims 1-18, wherein said specimen surface and/or said substrate surface is made of a flexible material.

20. The method as in any of claims 1-19, wherein said 35 substrate surface is a metal surface.

21. The method as in claim 20, wherein said metal is silver, gold, palladium, platinum, nickel, chromium, or copper, preferably silver.

5 22. The method as in any of claims 1-21, wherein said substrate surface is structured.

23. The method as in claim 22, wherein said substrate surface is structured with protrusions of 0.01-5  $\mu\text{m}$ .

24. The method as in any of claims 1-21, wherein said substrate surface is polished.

10 25. The method as in any of claims 1-24, wherein said substrate surface is cleaned immediately before said imprint is produced.

15 26. The method as in claim 25, wherein said substrate surface is cleaned by means of chemical etching, plasma cleaning, or vaporization deposition, or a combination thereof.

20 27. The method as in any of claims 1-26, wherein said specimen surface is subjected to lyophilization, freeze-substitution, or air drying before said imprint is produced.

28. The method as in any of claims 1-27, wherein said imprint is produced by pressing said specimen surface against said substrate surface.

25 29. The method as in claim 28, wherein said pressing is accomplished by means of a compressible material.

30 30. The method as in claim 28 or 29, wherein said pressing is accomplished by applying a force between 0.01 and 10 MPa.

31. The method as in any of claims 28-30, wherein said pressing is performed for up to 100 s.

32. The method as in any of claims 1-31, wherein said said pressing is performed so that said imprint represents below 5 monolayers, preferably below 2 monolayers, of said at least one chemical substance on said substrate surface.

33. The method as in any of claims 1-32, wherein said imaging mass spectrometry is a secondary ion mass spectrometry.

5 34. The method as in claim 33, wherein said secondary ion mass spectrometry is time-of-flight secondary ion mass spectrometry.

35. The method as in claim 33-34, wherein a focused beam of ions is produced by the primary ion source in said secondary ion mass spectrometry.

10 36. The method as in claim 35, wherein said ions are C<sub>60</sub>, Ga, In, or Au ions.

37. The method as in claim 36, wherein said Au ions are clusters of n ions, n ≤ 10.

15 38. The method as in any of claims 35-37, wherein said focused beam has a diameter below 10 μm, preferably below 1 μm.

39. The method as in any of claims 1-32, wherein a light sensitive matrix is applied onto said substrate surface before said imprint is produced.

20 40. The method as in any of claims 1-32 and 39, wherein a light sensitive matrix is applied onto said substrate surface after said imprint is produced.

25 41. The method as in any of claims 1-32 and 39-40, wherein said imaging mass spectrometry is matrix assisted laser desorption ionisation.

42. The method as in claim 41, wherein the light source of said matrix assisted laser desorption ionization comprises a focused laser beam, preferably an ultraviolet laser beam.

30 43. The method as in any of claims 1-42, wherein said at least one signal is recorded from an array of points on said substrate surface.

44. The method as in any of claims 1-43, wherein said at least one image is produced from said at least one signal, the colour or the brightness in each point of said

at least one image being dependent on the magnitude of said at least one signal from the corresponding point on said substrate surface.

## ABSTRACT

A method of analyzing the spatial distribution of at least one chemical substance retained by a biological matter comprises the steps of

5 (a) supplying a sample of said biological matter as a specimen surface;

(b) producing an imprint of said specimen surface on a substrate surface, said at least one chemical substance

10 being distributed on the same;

(c) subjecting said imprint to imaging mass spectrometry, at least one signal from at least one point of said substrate surface being produced, the magnitude of said at least one signal being dependent on the amount of said at

15 least one chemical substance present on said substrate surface;

(d) recording said at least one signal; and

(e) determining said distribution of said at least one chemical substance from at least one image of said imaging

20 mass spectrometry.